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The Dynamic Expression Pattern of *frzb-1* Suggests Multiple Roles in Chick Development

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The Wnt family of secreted proteins has been shown to have multiple roles in embryonic development. Wnt signals are thought to be propagated by binding to the cysteine-rich extracellular domain (CRD) of Frizzled, a seven-transmembrane-domain cell surface receptor. Secreted Frizzled-related proteins (generally denoted Frzb or Sfrp) possess a domain with a high degree of sequence identity and structural similarity with the CRD of Frizzled. Current data indicate that the cysteine-rich domain of secreted Frzb proteins can bind Wnt proteins, suggesting the possibility that Frzbs compete with membrane-bound Frizzled for Wnt binding and consequently act as competitive inhibitors of Wnt signaling. In order to gain a better understanding of the potential roles of Frzb-1 in chick development, we utilized the polymerase chain reaction to isolate a partial cDNA of the chick orthologue of *frzb-1*, *cfrzb-1*, and compared its expression pattern to that of *Wnt-1*, *Wnt-3a*, *Wnt-5a*, *Wnt-7a*, and *Wnt-8c*. Whole-mount *in situ* hybridizations have revealed three major phases of expression for *cfrzb-1* in the developing chick. The earliest expression of *cfrzb-1* is in cells fated to become neural ectoderm in streak-stage embryos. Expression of *cfrzb-1* in the neural ectoderm continues up through stage 8. After stage 8, *cfrzb-1* expression is gradually attenuated in the closing neural tube of the trunk and is concomitantly up-regulated in neural crest cells. Finally, *cfrzb-1* appears in the condensing mesenchyme of the bones in both the limb and the trunk in stage 25+ embryos. Comparative analysis of the *cfrzb-1* and the *Wnt* gene expression patterns suggests possible interactions between cFrzb-1 and all of the Wnt family members examined. © 2000 Academic Press

Key Words: **chick; chondrogenesis; development; embryo; Frizzled; Frzb; gastrulation; Hensen's node; HNK-1, limb; neural crest; neurulation; Sarp; Sfrp; whole-mount *in situ* hybridization; Wnt.**

INTRODUCTION

The Wnt family of secreted signaling proteins has been implicated in a variety of early embryonic events during vertebrate development, including gastrulation (Hume and Dodd, 1993; Takada *et al.*, 1994; Yamaguchi *et al.*, 1999), somite patterning (Capdevila *et al.*, 1998; Hirsinger *et al.*, 1997; Ikeya and Takada, 1998; Marcelle *et al.*, 1997; Reshef *et al.*, 1998), myogenesis (Ikeya and Takada, 1998; Münsterberg *et al.*, 1995; Stern *et al.*, 1995; Tajbakhsh *et al.*, 1998), chondrogenesis (Kawakami *et al.*, 1999; Rudnicki and Brown, 1997; Stott *et al.*, 1999), kidney development (Kispert *et al.*, 1998; Stark *et al.*, 1994), tooth development (Dássùle and McMahon, 1998; Sarkar and Sharpe, 1999), hematopoiesis (Austin *et al.*, 1997; Van Den Berg *et al.*, 1998), limb development (Kawakami *et al.*, 1999; Kengaku *et al.*, 1998; Parr and McMahon, 1995; Yamaguchi *et al.*, 1999; Yang and Niswander, 1995), craniofacial development (Augustine *et al.*, 1993), gonad development/sex determination (Miller *et al.*, 1998; Miller and Sassoon, 1998; Vainio *et al.*, 1999), and the proper establishment of the central and peripheral nervous systems (Chang and Hemmati-Brivanlou, 1998; Dorsky *et al.*, 1998; Ikeya *et al.*, 1997; LaBonne and Bronner-Fraser, 1998; McMahon and Bradley, 1990; Saint-Jeannet *et al.*, 1997; Takada *et al.*, 1994; Thomas and Capecchi, 1990). As Wnt proteins are important regulators of cellular proliferation, specification, and adhesion (for review, see Cadigan and Nusse, 1997), other proteins that modify Wnt signals are likely to play an equally important role in these cellular and developmental processes.

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Wnt signals are thought to be transduced by the Frizzled family of proteins (Bhanot *et al.*, 1996; He *et al.*, 1997). Frizzled receptors possess seven membrane spanning domains and a cysteine-rich extracellular domain, which is sufficient for binding of Wnt to Frizzled (He *et al.*, 1997). The recent identification of a family of at least seven secreted frizzled-related proteins (Frzb/Sfrp/Sarp), which possess a high degree of sequence identity and structural similarity to the cysteine-rich domain of frizzled receptors, suggests a potential for these molecules to associate with Wnt proteins and, hence, modulate the activity of Wnt proteins (Chang *et al.*, 1999; Finch *et al.*, 1997; Hoang *et al.*, 1996; Hu *et al.*, 1998; Leimeister *et al.*, 1998; Lescher *et al.*, 1998; Leyns *et al.*, 1997; Lin *et al.*, 1997; Mayr *et al.*, 1997; Melkonyan *et al.*, 1997; Pfeffer *et al.*, 1997; Rattner *et al.*, 1997; Salic *et al.*, 1997; Shirozu *et al.*, 1996; Wang *et al.*, 1997a; Wolf *et al.*, 1997; Xu *et al.*, 1998; Zhou *et al.*, 1998). Several studies have demonstrated that the expression patterns of *Wnt* genes and *frzb* genes are both complementary and overlapping during embryonic development (Hoang *et al.*, 1998; Lescher *et al.*, 1998), further suggesting the possibility that Wnts and Frzbs may interact during development.

Although Frzb proteins have been shown to bind Wnt proteins *in vitro* (Lescher *et al.*, 1998; Leyns *et al.*, 1997; Lin *et al.*, 1997; Rattner *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997a), the type(s) of signal modulation that occurs *in vivo* is still unclear. Frzb-1 can bind to and inhibit Wnt-1 and Wnt-8 signaling in *Xenopus* (Finch *et al.*, 1997; Leyns *et al.*, 1997; Lin *et al.*, 1997; Mayr *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997a; Xu *et al.*, 1998), while it can bind Wnt-5a (Lin *et al.*, 1997) without apparent inhibition of signaling (Lin *et al.*, 1997; Wang *et al.*, 1997b). Although the failure of Frzb-1 to inhibit the activity of Wnt-5a could be due to the addition of insufficient concentrations of Frzb-1 protein, this result also raises the possibility that binding of Frzb-1 does not necessarily result in inhibition of Wnt signaling. In fact, one could easily envision agonistic roles for Frzb-1. If Wnts have a lower affinity for Frzb than Frizzled, then Frzb may play a role in sequestering Wnts in particular areas and then releasing them for binding to Frizzled proteins. Alternatively, Frzb could serve as a cofactor for binding to Frizzled. Or, it may even help escort Wnt proteins, which are poorly secreted in heterologous systems (Bradley and Brown, 1990; Burrus and McMahon, 1995; Papkoff *et al.*, 1987), through the secretory pathway.

To date, there is more evidence to support an antagonistic role for Frzb than an agonistic role, but the two roles are not necessarily mutually exclusive. Sarp-1 (also called Sfrp-2) protects MCF7 breast adenocarcinoma cells from apoptosis while Sarp-2 (Sfrp-1) sensitizes cells to apoptotic agents (Melkonyan *et al.*, 1997). Sarp-1 is known to bind to Wnt-4 (Lescher *et al.*, 1998), and heterologous expression of Sarps alters levels of intracellular β-catenin (Melkonyan *et al.*, 1997), a downstream molecule in the Wnt signaling cascade (for review, see Willert and Nusse, 1998), indicating that Sarp proteins may either positively or negatively modulate the Wnt signaling pathway. Evidence for an antagonistic role for Frzb-1 is twofold. Whereas Wnt-1, Wnt-5a, and Wnt-7a inhibit chondrogenic differentiation (Kawakami *et al.*, 1999; Rudnicki and Brown, 1997; Stott *et al.*, 1999), Frzb-1 promotes chondrogenesis (Hoang *et al.*, 1996). As Frzb-1 has been shown to bind to Wnt-1 (Leyns *et al.*, 1997; Lin *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997a), this suggests that the role of Frzb proteins is to antagonize Wnt signaling by competing with Frizzled receptors for binding. This argument is further strengthened by experiments in *Xenopus* in which injection of RNA encoding various Frzb family members into embryos blocks both XWnt-8 and Wnt-1 activity (Finch *et al.*, 1997; Leyns *et al.*, 1997; Mayr *et al.*, 1997; Salic *et al.*, 1997; Wang *et al.*, 1997a; Xu *et al.*, 1998).

The potential importance of Wnt signaling and Wnt/Frzb interactions during early embryonic development along with the experimental advantages of the chick model system led us to search for the chicken orthologue of *frzb-1*. In order to identify possible roles for cFrzb-1 protein, we have characterized the expression pattern *cfrzb-1* transcripts. Whole-mount *in situ* hybridizations reveal transcripts in the neural ectoderm of early chicken embryos (stages 3–8). During later stages of development (stages 8–30), expression in the neural tube is attenuated and *cfrzb-1* is predominantly found in neural crest cell-derived lineages. In addition, we find distinct *cfrzb-1* expression in developing bones. Comparison of the expression pattern of *cfrzb-1* with the expression patterns of *Wnt-1, Wnt-3a, Wnt-5a, Wnt-7a* and *Wnt-8c* indicates that these genes are expressed in complementary patterns at most stages of development. Cumulatively, these data lead us to propose that cFrzb-1 plays a role in gastrulation, patterning of the peripheral nervous system, and chondrogenesis, perhaps by antagonizing local Wnt signals.

MATERIALS AND METHODS

Materials

Materials and their respective vendors are as follows: GeneClean II Kit (Bio101); avian myeloblastosis virus (AMV) reverse transcriptase, anti-digoxigenin F_{ab}, blocking reagent, BM purple, goat anti-mouse secondary antibody conjugated with horseradish peroxidase, Triton X-100, TriPure isolation reagent, T_4 DNA ligase, T7 RNA polymerase (Boehringer Mannheim); Fibrowax paraffin (Gallard Schlesinger Industries); goat anti-mouse IgG conjugated with TRITC (Jackson Immunoresearch); Slowfade Light (Molecular Probes); oligonucleotide primers (Operon); *Taq* polymerase (Perkin–Elmer); miniprep spin columns (Qiagen); and pBluescript II KS($^+$) (Stratagene). HNK-1 antibodies were generously provided by Dr. Carol Erickson (UC Davis) and the chick *Wnt-1, Wnt-3a,* and *Wnt-5a* cDNAs were a gift from Dr. Andy McMahon (Harvard University). The *Wnt-7a, Wnt-8c,* and *sox-9* cDNAs were kindly provided by Dr. Tony Brown (Cornell University Medical College), Dr. Jane Dodd (Columbia University), and Dr. Chris Healy/Dr. Paul Sharpe (Guy's Hospital, London), respectively.

Fertilized White Leghorn chicken eggs (*Gallus gallus domesti-*

cus) were obtained from Featherhill Farms (Petaluma, CA) and incubated in a 38°C humidified incubator. Embryos were staged according to Hamburger and Hamilton (1951).

Isolation of cfrzb-1 Partial cDNA

Total RNA was isolated from stage 10 embryos using a monophasic solution of phenol and guanidine thiocyanate (TriPure isolation reagent). For each 75 mg of tissue, 1 ml of TriPure solution was used. Embryos were homogenized in the TriPure solution using a polytron homogenizer and stored at −80°C. Total RNA was isolated by adding 0.2 vol chloroform to the TriPure/tissue solution. After thorough mixing, the solution was centrifuged and the aqueous phase was isolated. RNA was precipitated by adding 0.5 vol isopropanol and microfuging 15 min at 12,000*g*. RNA pellets were washed with 75% EtOH resuspended in DEPC-treated water and stored at −80°C. Total RNA (1 μg) was reverse transcribed with 20 units of AMV reverse transcriptase in 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM dNTPs, 80 μg/ml oligo(dT) primer, 2.5 Units/μl RNasin, and 0.01 μg/μl gelatin in a total volume of 20 μl for 1 h at 42°C.

Degenerate primers (Operon) for *cfrzb-1* (upstream—5′-GTGAATTCTGGAAYATGACNAARATG-3′; downstream—5′-GTACTAGTCYTCRTANCCCATDAT-3′) corresponding to amino acid sequences WNMTKM and IMGYED, respectively, were used (D = A/G/T; R = A/G; Y = C/T; N = A/C/G/T). Primer sequences were based on conserved sequences of Frzb homologs identified with PILEUP software provided by Genetics Computer Group (GCG, 1994). Restriction endonuclease sites for *Spe*I and *Eco*RI were engineered into the primers for subcloning.

Amplification of the cDNA product (4 μl) was carried out through 35 cycles of PCR with an annealing temperature of 50°C. Reactions were carried out in a final volume of 25 μl in a Perkin–Elmer 9700 thermocycler. Final concentrations of reagents in the reaction were as follows: 3 mM Tris (pH 8.3), 40 mM KCl, 1.25 mM $MgCl_2$, 180 μM dNTPs, 10 μM each primer, and 3 Units *Taq* DNA polymerase.

Subcloning and Sequence Analysis of cfrzb-1

PCR products were purified using the Bio-101 GeneClean II kit and digested with *Spe*I and *Eco*RI for subcloning into pBluescript II KS(+). In order to rule out the possibility of PCR-induced sequence errors, both strands of four independent cDNA clones were sequenced in order to generate a consensus sequence. DNA was prepared by purification with a Qiagen spin miniprep column and both strands were sequenced using dideoxy dye terminators and analyzed using the ABI 670 sequencer and accompanying software. Chromatograms were analyzed using Sequencher 3.0 software and a consensus sequence was obtained. Sequence comparisons to other species were performed using software provided by the Genetics Computer Group (GCG, 1994).

Whole-Mount in Situ Hybridizations

Digoxigenin-labeled antisense probes for *cfrzb-1*, *Wnt-1*, and *Wnt-5a* were made by transcribing *Eco*RI-linearized cDNA with T7 RNA polymerase. *In situ* hybridization was carried out essentially as described by Parr and Wilkinson (Parr *et al.*, 1993; Wilkinson, 1995). Embryos were harvested in cold phosphate-buffered saline (PBS), and extraembryonic tissue was removed from embryos older than HH stage 12. The hindbrain and nasopharyngeal process were punctured to avoid background staining. Embryos were fixed overnight in PBS (11.5 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) containing 4% paraformaldehyde and dehydrated into 100% MeOH. Prior to use, embryos were rehydrated into PBS containing 0.1% Tween, and embryos older than HH stage 14 were treated with 6% H_2O_2 for 1 h. All embryos older than HH stage 12 were treated with 10 μg/ml proteinase K for 6–30 min. The reaction was quenched by the addition of 2 mM glycine. Embryos were then postfixed in 4% paraformaldehyde and 0.2% glutaraldehyde. Hybridization was done at 70°C in hybridization buffer (50% formamide, 1% SDS, 100 μg/ml heparin, 50 μg/ml tRNA) with 5× SSC (1× SSC contains 150 mM NaCl). Embryos were washed three times for 1 h at 70°C temperature in wash buffer containing 50% formamide, 5× SSC, and 1% SDS and an additional three times in buffer containing 50% formamide, 2× SSC, and 1% SDS.

Prior to antibody binding, embryos were preblocked in MABT (100 mM maleic acid, 150 mM NaCl, pH 7.5 with Tris base, 0.1% Tween 20) with 2% Boehringer Mannheim blocking reagent and 10% sheep serum. Anti-digoxigenin AP Fab fragments were preadsorbed with 6 mg/ml heat-treated "chick powder" and 10 μl/ml sheep serum at a 1:500 dilution of antibody. The antibody was then diluted to 1:2000 total dilution in MABT, 2% BM blocking reagent, and 1% sheep serum. Antibody binding was performed at 4°C, overnight. Embryos were washed six times (1 h each) in 1× MABT with a final wash that extended overnight at 4°C.

Embryos were then equilibrated in NTMT (100 mM Tris, pH 9.5, 50 mM $MgCl_2$, 100 mM NaCl, and 1% Tween 20) at room temperature. Embryos were stained at room temperature for 36–72 h with BM purple. Embryos were then washed in PBT and postfixed in 4% paraformaldehyde and 0.2% glutaraldehyde. Embryos were photographed in either PBT containing 80% glycerol or PBT alone using an Olympus SCR35 camera mounted on an Olympus SZX12 stereoscope.

HNK-1 Antibody Whole Mounts

Embryos were harvested, fixed, and dehydrated as described in the previous section. Embryos were then rehydrated in methanol and PBS with 0.5% Triton X, then washed in PBS and 0.5% Triton X to remove all traces of methanol. To reduce any endogenous peroxidase activity, embryos were then treated with 0.5% H_2O_2 for 1 h. Embryos were then preblocked for 3 h in PBS containing 5% heat-inactivated sheep serum, 0.2% BSA, and 0.5% Triton X. Following blocking, embryos were treated overnight at 4°C with a 1:5 dilution of HNK-1 antibody from hybridoma supernatant in 1% heat-inactivated sheep serum and 0.1% BSA. To remove unbound antibody, embryos were washed for 6 h at room temperature in PBS with 0.5% Triton X and 0.2% BSA. Goat anti-mouse Ig conjugated to horseradish peroxidase was used as the secondary antibody. Conditions for preblocking, antibody binding, and postbinding washes were identical to those used with the primary antibody except that the antibody dilution was 1:100. The embryos were then washed in PBS + 0.5% Triton X to remove BSA. For detection, embryos were incubated in diaminobenzidine (DAB) at 0.5 mg/ml for 10 min. Thirty percent H_2O_2 was added at 0.1% (v/v) with the DAB solution, and embryos were monitored until staining was complete. Embryos were then washed in PBT and postfixed in 4% paraformaldehyde and 0.2% glutaraldehyde, then stored in PBT at 4°C. Inclusion of 0.003% (w/v) $NiCl_2$ during the incubation with substrate resulted in a black stain rather than brown.

Sectioning

Embryos were washed in PBS to remove any Tween 20, then dehydrated in ethanol though a series of ethanol/PBS washes. Once in 100% ethanol, embryos were transferred xylene for 30 min, then equilibrated in Fibrowax overnight at 60°C. Embryos were embedded and sectioned on a Spencer 820 microtome, taking 15- to 40-μm sections. Sections were dried, dewaxed, and mounted in 80% glycerol for photography on a Nikon E600 compound microscope with a Spot Camera made by Diagnostic Instruments.

Immunolabeling of Sections with HNK-1

Immunolabeling of sections with HNK-1 antibodies was performed essentially as described by Reedy *et al.* (1998). Embryos were first subjected to whole-mount *in situ* hybridization as described above, except that glutaraldehyde was omitted from the protocol. Embryos labeled for *cfrzb-1* expression were equilibrated in PBS containing 5% sucrose and 0.01% sodium azide for 2 h at room temperature, then in PBS containing 15% sucrose and 0.01% sodium azide for 12 h at 4°C. Embryos were then incubated for 2 h at 37°C in PBS containing 15% sucrose and 7.5% gelatin, set in cryomolds, and frozen in liquid nitrogen. Sections (40 μm) were cut at −19°C in a Leica CM1800 cryostat.

Sections were washed once for 5 min at 50°C in PBS with 0.1 M glycine in order to solubilize residual gelatin, then for 5 min at room temperature in the same solution, and finally for 5 min in PBS alone. Nonspecific binding sites were blocked for 15 min with PBS containing 1% bovine serum albumin (BSA). Slides were incubated 12 h at 4°C with or without (for negative control) undiluted HNK-1 hybridoma supernatant and then washed three times in PBS (5, 10, and 15 min) to remove unbound primary antibody. Prior to addition of the secondary antibody, slides were blocked again for 15 min in PBS containing 1% BSA. Slides were then incubated 90 min at room temperature with 12.5 μg/ml goat anti-mouse IgG conjugated with TRITC in PBS containing 1% BSA, washed as above, and then mounted in Slowfade Light. Sections were photographed on a Nikon E600 compound microscope with a Spot Camera from Diagnostic Instruments.

RESULTS

Isolation of a Partial cDNA Encoding Chick frzb-1

In order to isolate a partial cDNA of the chick orthologue of *frzb-1*, a comparison of known anuran, bovine, and murine Frzb-1 (Sfrp-3) protein sequences was done to identify conserved sequences. From these conserved sequences, degenerate primers were designed, and the partial cDNA was amplified from total RNA of stage 10 embryos using RT-PCR. From this amplification, a 640-bp partial cDNA was isolated and four independent clones were sequenced (Fig. 1A). The predicted protein sequence is most closely related to bovine Frzb-1 and mouse Sfrp-3, sharing 88% identity with both (Fig. 1B).

Expression Pattern of Chick frzb-1 in Gastrulating Embryos

Antisense digoxigenin-labeled RNA probes were used to detect mRNA expression patterns by whole-mount *in situ* hybridization. Expression of *cfrzb-1* is seen in every stage of development analyzed, starting with Hamburger and Hamilton stage 3 and continuing through stage 30 (Hamburger and Hamilton, 1951). At streak stages, *cfrzb-1* is expressed in ectoderm which is fated to become neural plate (Garcia-Martinez *et al.*, 1993), but is absent from the primitive streak (Fig. 2A). Transverse sections confirm that *cfrzb-1* is predominantly expressed in the condensed epithelium rostral and lateral to the streak, with possible faint expression in the mesoderm (Fig. 2E). The faint staining in the mesoderm could reflect either extremely low levels of *cfrzb-1* in the mesoderm or bleedthrough of the label from the more darkly labeled ectoderm. In contrast to the expression pattern of *cfrzb-1*, *Wnt-3a* and *Wnt-8c* (Hume and Dodd, 1993) are expressed in the superficial and middle layers of the primitive streak (Figs. 2C, 2D, 2G, and 2H), while the expression of Wnt-*5a* is limited the middle layer of the streak (Figs. 2B and 2F).

cfrzb-1 and *Wnt-5a* continue to be expressed in complementary domains in and around the streak throughout early chick development. Because the embryo matures in a rostral to caudal progression, several stage-related changes in the expression of *cfrzb-1* and *Wnt-5a* can be compared in embryos of the same developmental stage by examining progressively more rostral levels or by examining the same level in progressively older embryos. Consistent with the expression patterns of *cfrzb-1* and *Wnt-5a* in the gastrula stage, *cfrzb-1* continues to be excluded from the streak (Figs. 3B and 3H) in stage 8/9 embryos, whereas *Wnt-5a* is expressed in the mesoderm ingressing through the streak and in mesoderm lateral to the streak (Figs. 3C and 3I). Just rostral to the node, *cfrzb-1* is clearly expressed in the neural plate and neural folds (Figs. 3B and 3F), whereas *Wnt-5a* is expressed in the notochord and lateral mesoderm (Figs. 3C and 3G). By stage 10 of development, *cfrzb-1* expression is also observed in the lateral plate mesoderm (Fig. 5B). In the cranial region where neural tube closure has occurred, *cfrzb-1* becomes restricted to the dorsal neural tube or prospective neural crest (Figs. 3B and 3D), while *Wnt-5a* is no longer expressed (Fig. 3C). Similar patterns of expression are evident in embryos labeled at stage 12 (Figs. 6E–6J) with the progression shifted caudally as the neural tube closes and the node regresses in this direction.

Expression of cfrzb-1 in Neural Crest Cells

Preliminary analysis of *cfrzb-1* expression in stage 8 to 12 embryos, which were labeled in whole mount, indicated that the timing and position of the expression of *cfrzb-1* in cranial regions were consistent with the possibility that *cfrzb-1* is expressed in neural crest cells (see Figs. 3–6). To test for this possibility, we have compared the expression of *cfrzb-1* with the binding pattern of HNK-1, a well-characterized antibody that recognizes a carbohydrate epitope found on migrating neural crest cells (Bronner-Fraser, 1986; Kuratani, 1991; Tucker *et al.*, 1984, 1988). In stage 10/11 embryos, *cfrzb-1* expression is darkest in cells

A

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          1                                                   50
bfrzb1    MVCGSRGGML LLPAGLLALA ALCLLRVPGA RAAACEPVRI PLCKSLPWNM
hfrzb1    MVCGSPGGML LLRAGLLALA ALCLLRVPGA RAAACEPVRI PLCKSLPWNM
cfrzb1    .......... .......... .......... .......... .......WNM
msfrp3    MVCCGPGRML LGWAGLLVLA ALCLLQVPGA QAAACEPVRI PLCKSLPWNM
xfrzb1    ....MSPTRK LDSFLLLVIP GLVLLLLPNA YCASCEPVRI PMCKSMPWNM

          51                                                  100
bfrzb1    TKMPNHLHHS TQANAILAIE QFEGLLGTHC SPDLLFFLCA MYAPICTIDF
hfrzb1    TKMPNHLHHS TQANAILAIE QFEGLLGTHC SPDLLFFLCA MYAPICTIDF
cfrzb1    TKMPNHLHHS TQANAVLAME QFEGLLGTNC SPDLLFFLCA MYAPICTIDF
msfrp3    TKMPNHLHHS TQANAILAME QFEGLLGTHC SPDLLFFLCA MYAPICTIDF
xfrzb1    TKMPNHLHHS TQANAILAIE QFEGLLTTEC SQDLLFFLCA MYAPICTIDF

          101                                                 150
bfrzb1    QHEPIKPCKS VCERARQGCE PILIKYRHSW PESLACEELP VYDRGVCISP
hfrzb1    QHEPIKPCKS VCERARQGCE PILIKYRHSW PENLACEELP VYDRGVCISP
cfrzb1    QHEPIKPCKS VCERARAGCE PVLIRYRHAW PESLACDELP LYDRGVCISP
msfrp3    QHEPIKPCKS VCERARQGCE PILIKYRHSW PESLACDELP VYDRGVCISP
xfrzb1    QHEPIKPCKS VCERARAGCE PILIKYRHTW PESLACEELP VYDRGVCISP

          151                                                 200
bfrzb1    EAIVTADGA. ....DFPMDS SNGNCRGASS ERCKCKPVRA TQKTYFRNNY
hfrzb1    EAIVTADGA. ....DFPMDS SNGNCRGASS ERCKCKPIRA TQKTYFRNNY
cfrzb1    EAIVTAEGA. ....DFPMDS NNGNCGGTGI ERCKCKPIKA TQKTYVRNNY
msfrp3    EAIVTADGA. ....DFPMDS STGHCRGASS ERCKCKPVRA TQKTYFRNNY
xfrzb1    EAIVTVEQGT DSMPDFPMDS NNGNCGSTAG EHCKCKPMKA SQKTYLKNNY

          201                                                 250
bfrzb1    NYVIRAKVKE IKTKCHDVTA VVEVKEILKA SLVNIPRETV NLYTSSGCLC
hfrzb1    NYVIRAKVKE IKTKCHDVTA VVEVKEILKS SLVNIPRDTV NLYTSSGCLC
cfrzb1    NYVIRAKVKE VKTKCHDVTA VVEVKEILKS SLVNIPKDTV NLYTNSGCLC
msfrp3    NYVIRAKVKE VKMKCHDVTA VVEVKEILKA SLVNIPRDTV NLYTTSGCLC
xfrzb1    NYVIRAKVKE VKVKCHDATA IVEVKEILKS SLVNIPKDTV TLYTNSGCLC

          251                                                 300
bfrzb1    PPLNVNEEYL IMGYEDEERS RLLLVEGSIA EKWKDRLGKK VKRWDMKLRH
hfrzb1    PPLNVNEEYI IMGYEDEERS RLLLVEGSIA EKWKDRLGKK VKRWDMKLRH
cfrzb1    PPLSANEEYI IMGYED.... .......... .......... ..........
msfrp3    PPLTVNEEYV IMGYEDEERS RLLLVEGSIA EKWKDRLGKK VKRWDMKLRH
xfrzb1    PQLVANEEYI IMGYEDKERT RLLLVEGSLA EKWRDRLAKK VKRWDQKLRR

          301                                 331
bfrzb1    LGLNTSDSSH SDSTQSQKPG RNSNSRQARN *
hfrzb1    LGLSKSDSSN SDSTQSQKSG RNSNPRQARN *
cfrzb1    .......... .......... .......... .
msfrp3    LGLGKTDA.. SDSTQNQKSG RNSNPRPARS *
xfrzb1    .......PRK SKDPVAPIPN KNSNSRQARS *
```

B

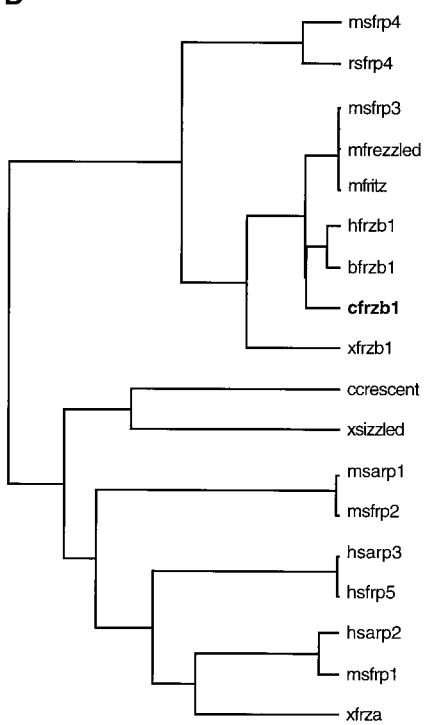

FIG. 1. Comparison of amino acid sequences of cFrzb-1 and other homologues. (A) Alignment of our deduced amino acid sequence with Frzb-1 orthologues from mouse, frog, cow, and human. (B) Dendrogram of Frzb family members (Chang *et al.,* 1999; Finch *et al.,* 1997; Hoang *et al.,* 1996; Hu *et al.,* 1998; Leimeister *et al.,* 1998; Lescher *et al.,* 1998; Leyns *et al.,* 1997; Mayr *et al.,* 1997; Melkonyan *et al.,* 1997; Pfeffer *et al.,* 1997; Rattner *et al.,* 1997; Salic *et al.,* 1997; Shirozu *et al.,* 1996; Wang *et al.,* 1997a; Wolf *et al.,* 1997; Xu *et al.,* 1998; Zhou *et al.,* 1998). Alignments were generated using the PILEUP application from the Genetics Computer Group (GCG, 1994).

lateral to the neural epithelium of the prosencephalon, the mesencephalon, and the anteriormost rhombencephalon (Fig. 5B). In the cranial region, the expression patterns of *cfrzb-1* and HNK-1 seen in whole mount are very similar except that *cfrzb-1* appears to be expressed in or dorsal to the neural tube, while HNK-1 is clearly excluded from the neural tube (Figs. 5A and 5B). In stage 12 embryos, HNK-1-positive cells can be seen migrating in streams just rostral and caudal to the otic pits (Fig. 6B). We also observe the streaming of HNK-1-positive cells from the rhombomeres into the branchial arches (Fig. 6B). It has previously been shown that HNK-1 labels neural crest cells migrating from rhombomeres 1, 2, 4, and 6, but not rhombomeres 3 and 5 (Kuratani, 1991). However, DiI labeling and grafting experiments have yielded disparate results as to whether rhombomeres 3 and 5 actually give rise to neural crest cells (Couly and Le Douarin, 1990; Lumsden *et al.,* 1991; Sechrist *et al.,* 1993). Our results show that *cfrzb-1* is clearly expressed in cells emerging from rhombomeres 1, 2, 4, and 6 (Figs. 5B and 6A). Although labeling is seen adjacent to rhombomere 5, we cannot be certain that the labeled cells actually emigrated from rhombomere 5. And, while no

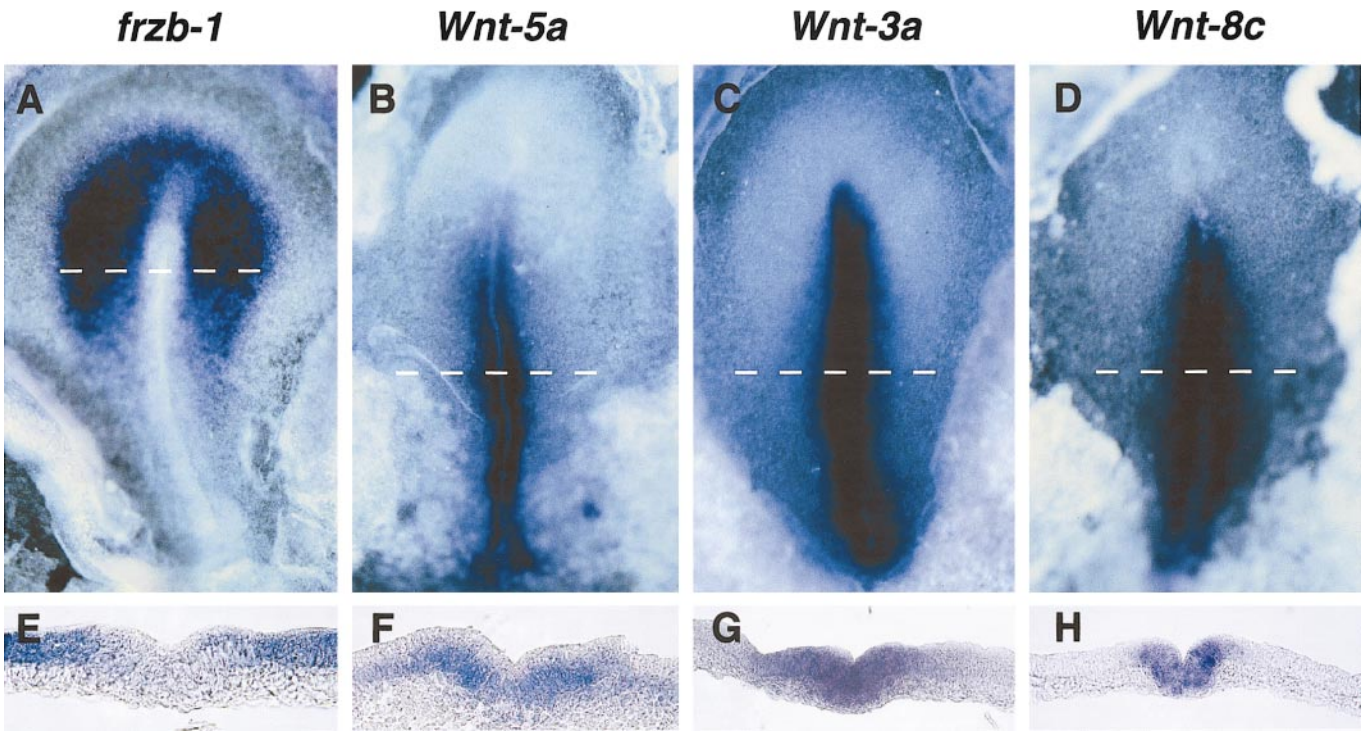

FIG. 2. Expression of *cfrzb-1*, *Wnt-3a*, *Wnt-5a*, and *Wnt-8c* in gastrula-stage chick embryos. Whole-mount *in situ* hybridization was used to detect expression of *cfrzb-1* (A), *Wnt-5a* (B), *Wnt-3a* (C), and *Wnt-8c* (D). Sections (15–20 μm) of embryos labeled in whole mount show expression of *cfrzb-1* in the presumptive neural ectoderm (E), *Wnt-5a* in the middle layer of the primitive streak (F), and *Wnt-3a* (G) and *Wnt-8c* (H) in both the middle and the superficial layers of the streak.

labeling is observed adjacent to rhombomere 3, we cannot eliminate the possibility that labeled cells are emigrating from the extreme rostral and caudal boundaries of rhombomere 3.

Whereas crest cells migrating from rhombomeres 1 and 2 migrate into the first branchial arch, those migrating from rhombomeres 4 and 6 populate arches 2 and 3, respectively (Lumsden *et al.*, 1991). In stage 19/20 embryos, neural crest cells have populated the branchial arches as evidenced by HNK-1 staining (Fig. 7A). Once again, *cfrzb-1* expression (Fig. 7B) closely resembles that of HNK-1. In contrast to earlier stages of development, *Wnt-5a* expression now overlaps with *cfrzb-1* in the branchial arches, limb buds, heart, otic vesicle, and eye (Figs. 7B and 7C).

The localization of *cfrzb-1* labeling in sections is also consistent with the possibility that *cfrzb-1* is expressed in neural crest cell lineages. In the cranial region of stage 8 embryos, *cfrzb-1* expression is localized to the dorsolateral edges of the neural tube (Fig. 3D). This staining may represent neural crest cells that are poised to emigrate from the neural tube or intracellular localization of *cfrzb-1* transcripts. Immunostaining of sections through the rhombencephalon of a stage 9 embryo demonstrates clear colocalization of *cfrzb-1* transcripts and HNK-1 epitopes in cells dorsal and lateral to the neural tube (Figs. 4B–4D). In stage 10/11 embryos, sections through the prosencephalon reveal both *cfrzb-1* and HNK-1 expression in crest-derived mesenchyme dorsal to the optic vesicles and in prenotochordal mesenchyme ventral to the neural tube (Figs. 5D and 5E). In the mesencephalon, HNK-1 and *cfrzb-1* are expressed in cells that are streaming away from the neural tube (Figs. 5G and 5H). And in the anterior rhombencephalon, HNK-1 is expressed in the thickened ectoderm of the otic placode (Fig. 5J), in the mesenchymal cells that are positioned both dorsally (Fig. 6D) and ventrally (Fig. 5J), and in the epimyocardium of the heart (Fig. 5J). It has recently been shown that cells committed to neural crest lineages contribute to the otic placode (Mayordomo *et al.*, 1998), suggesting that the expression of HNK-1 in the otic placode represents a crest-derived component of the otic placode. *cfrzb-1* is similarly expressed in the otic placode (Fig. 5K) as well as cells dorsolateral to the neural tube (Figs. 5H and 6D). Although analysis of stage 10/11 embryos labeled with *cfrzb-1* does not reveal any cardiac staining, we clearly observe HNK-1, *cfrzb-1*, and *Wnt-5a* expression in a variegated pattern in the heart by stage 19/20 of development (Figs. 7A–7C). Once again, in order to more rigorously determine if *cfrzb-1* is expressed in neural crest cells, we have performed a double-labeling experiment in

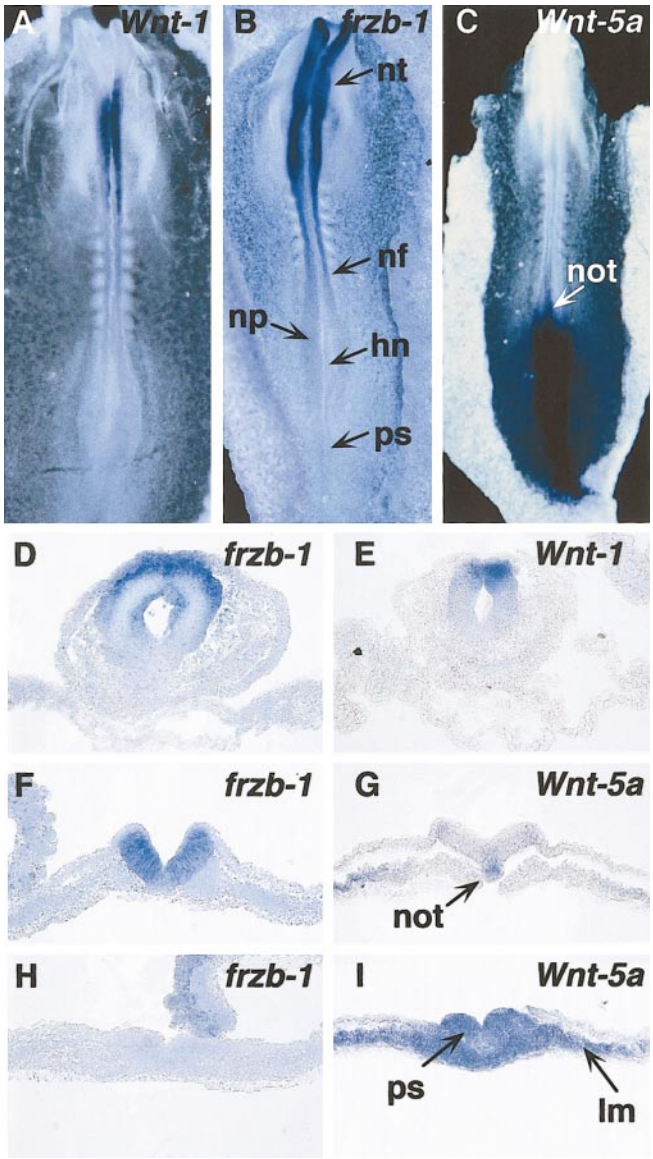

FIG. 3. Comparison of the expression patterns of *cfrzb-1*, *Wnt-1*, and *Wnt-5a* in stage 8/9 chick embryos. Whole-mount *in situ* hybridization was used to detect expression of *Wnt-1* (A), *cfrzb-1* (B), and *Wnt-5a* (C). Representative sections through the midbrain (D and E), the neural folds (F and G), and the primitive streak (H and I) are shown. *Cfrzb-1* (D) and *Wnt-1* (E) are expressed in distinct, but overlapping, domains in the dorsal neural tube. Rostral to Hensen's node, *cfrzb-1* (F) is expressed throughout the neural folds while *Wnt-5a* (G) is expressed in the notochord. No expression of *cfrzb-1* (H) is detected in the primitive streak while *Wnt-5a* (I) is expressed in the primitive streak and lateral mesoderm. Abbreviations: hn, Hensen's node; lm, lateral mesoderm, nf, neural fold; np, neural plate; nt, neural tube; not, notochord; ps, primitive streak.

which embryos that have been labeled in whole mount with antisense *cfrzb-1* probe were sectioned and immunostained with HNK-1 antibodies. In the anterior rhombencephalon, both *cfrzb-1* transcripts and HNK-1 epitopes are clearly present in cells dorsal and lateral to the neural tube.

Comparison of HNK-1- and *cfrzb-1*-labeled sections from stage 9–12 embryos demonstrates that unlike HNK-1, which is expressed exclusively in migratory crest cells, *cfrzb-1* is expressed in the dorsal neural tube as well as in cells positioned dorsal and lateral to the neural tube (Figs. 4–6). These data suggest that *cfrzb-1* may be expressed in both premigratory and migratory neural crest cells.

In the trunk, neural crest cells migrate in two distinct waves of migration (with an earlier wave also occurring in the vagal region; Bronner-Fraser, 1986; Loring and Erickson, 1987; Serbedzija *et al.*, 1989). Cells first migrate down a ventromedial pathway through the somite. Cells in this pathway give rise to a number of different lineages, including sensory neurons, glia, sympathoadrenal cells, and Schwann cells. Migration of crest cells down the ventromedial pathway occurs in a segmented fashion as crest cells are found only in the rostral portion of the somite (Bronner-Fraser, 1986; Loring and Erickson, 1987; Rickmann *et al.*, 1985). Additionally, migration of crest cells initiates at the cranial border of the somite and then proceeds caudally, giving rise to a characteristic triangular pattern. By stage 12, the first crest cells to migrate down the ventromedial pathway in the vagal region of the trunk are evident. HNK-1 staining at the axial level of the first several somites clearly exemplifies this typical pattern of migration (Fig. 6B). Sections of embryos stained in whole mount confirm these results, with the *cfrzb-1* pattern of expression recapitulating that of HNK-1. In older embryos (stages 19–30), HNK-1 stains crest cells that have migrated ventrally (Fig. 7D). Although in whole mount, *cfrzb-1* expression is easily visualized in nerve bundles (Fig. 7B), we have had difficulty visualizing *cfrzb-1* expression in interior ventral structures in sections (Fig. 7E). However, both HNK-1 and *cfrzb-1* are clearly expressed in the dorsal mesentery of stage 26 chick embryos (Figs. 8H and 8L), suggesting that *cfrzb-1* is indeed expressed in ventromedial neural crest cell lineages. Furthermore, when day 7 embryos were dissected and then subjected to whole-mount *in situ* hybridization, dorsal root ganglia and sensory and motor roots showed strong expression of *cfrzb-1* (Fig. 8G). As the cell bodies of sensory neurons are contained in the dorsal root ganglia and motor neurons are derived from the central nervous system, it is likely that the labeling of sensory and motor roots reflects expression in the Schwann cells surrounding the axons.

A second wave of migration commences down the dorsolateral pathway after ventromedial migration has been completed. These cells migrate in a nonsegmented fashion between the somite and the ectoderm and will give rise to pigment cells in the epidermal and dermal layers (Bronner-Fraser, 1986; Erickson *et al.*, 1992; Serbedzija *et al.*, 1989). Staining with HNK-1 reveals a punctate pattern on the surface of the embryo (Fig. 8E). Interestingly, we have not observed a similar pattern in *cfrzb-1*-labeled embryos (Fig. 8F). Although this could reflect damage to the ectoderm by

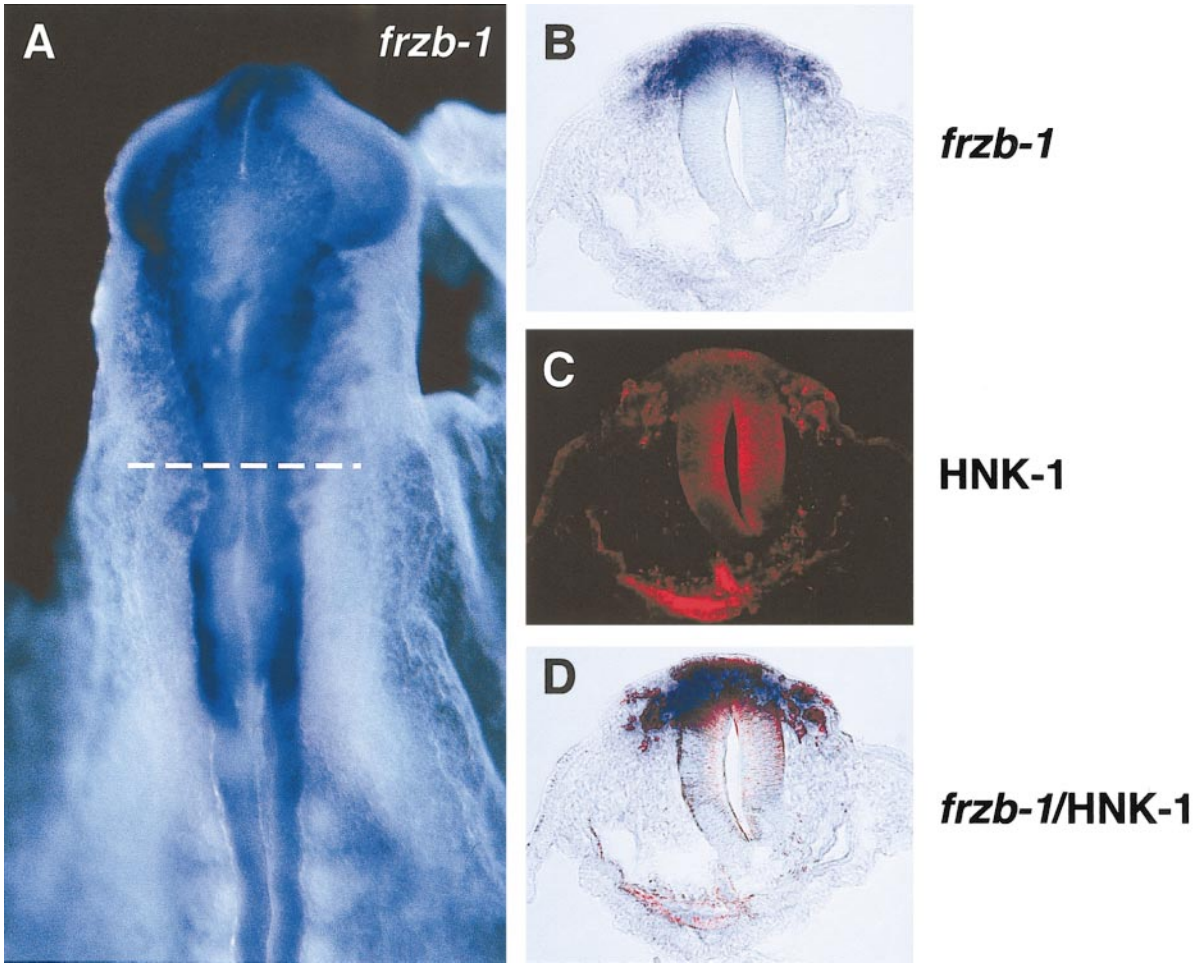

FIG. 4. Colocalization of *cfrzb-1* transcripts and HNK-1 epitopes in the rhombencephalon of a stage 9 embryo. The embryo was labeled in whole mount by *in situ* hybridization with an anti-sense *cfrzb-1* probe. In A, *cfrzb-1*-labeled cells are seen migrating away from the neural tube. The same embryo was cryosectioned and subjected to immunostaining with HNK-1 antibodies. The level of the section shown in B, C, and D is denoted by the white dashed line in A. In B, a bright-field micrograph of the *cfrzb-1* labeling in the dorsal neural tube and in cells lateral to the neural tube is shown. In C, a fluorescent micrograph of the HNK-1 staining is shown. As the HNK-1 antibody does not exclusively bind neural crest cells, it is not surprising to see other staining such as that in the neural tube. The images in B and C were digitally merged to give the composite figure shown in D.

proteinase K, reducing or omitting the proteinase K treatment fails to reveal *cfrzb-1* expression in a pattern that resembles HNK-1 expression in migrating dorsolateral crest, indicating that *cfrzb-1* may be excluded from this neural crest cell population.

Although we have not seen punctate labeling that would be consistent with the expression of *cfrzb-1* in migrating pigment cells, we do observe faint expression of *cfrzb-1* transcripts in the dermis starting as early as stage 14 (see Fig. 7E) and continuing up through at least stage 28. As dorsolateral crest cells begin to migrate at approximately stage 20 of development (Erickson *et al.*, 1992), the timing of this dermal expression suggests that it is not likely to represent labeling of dorsolateral crest cells.

As Wnt-1 has previously been shown to play important roles in neural crest cell proliferation and specification, we have also compared the expression pattern of *cfrzb-1* to that of *Wnt-1*. In stage 8 embryos, *Wnt-1* is expressed in an overlapping, but nonidentical pattern in the dorsal neural tube (Fig. 3A). Consistent with previously published data, *Wnt-1* expression begins in the dorsal and lateral walls of the mesencephalon and in the dorsal aspect of the neural folds in the rhombencephalon and spinal cord (Figs. 3A and 3E; Hollyday *et al.*, 1995). With the exception of the mesencephalic expression, *Wnt-1* expression is limited to the dorsal tips of the closing neural folds and the roof plate of the closed neural tube. At trunk levels of stage 10 embryos, both whole mounts and sections clearly demon-

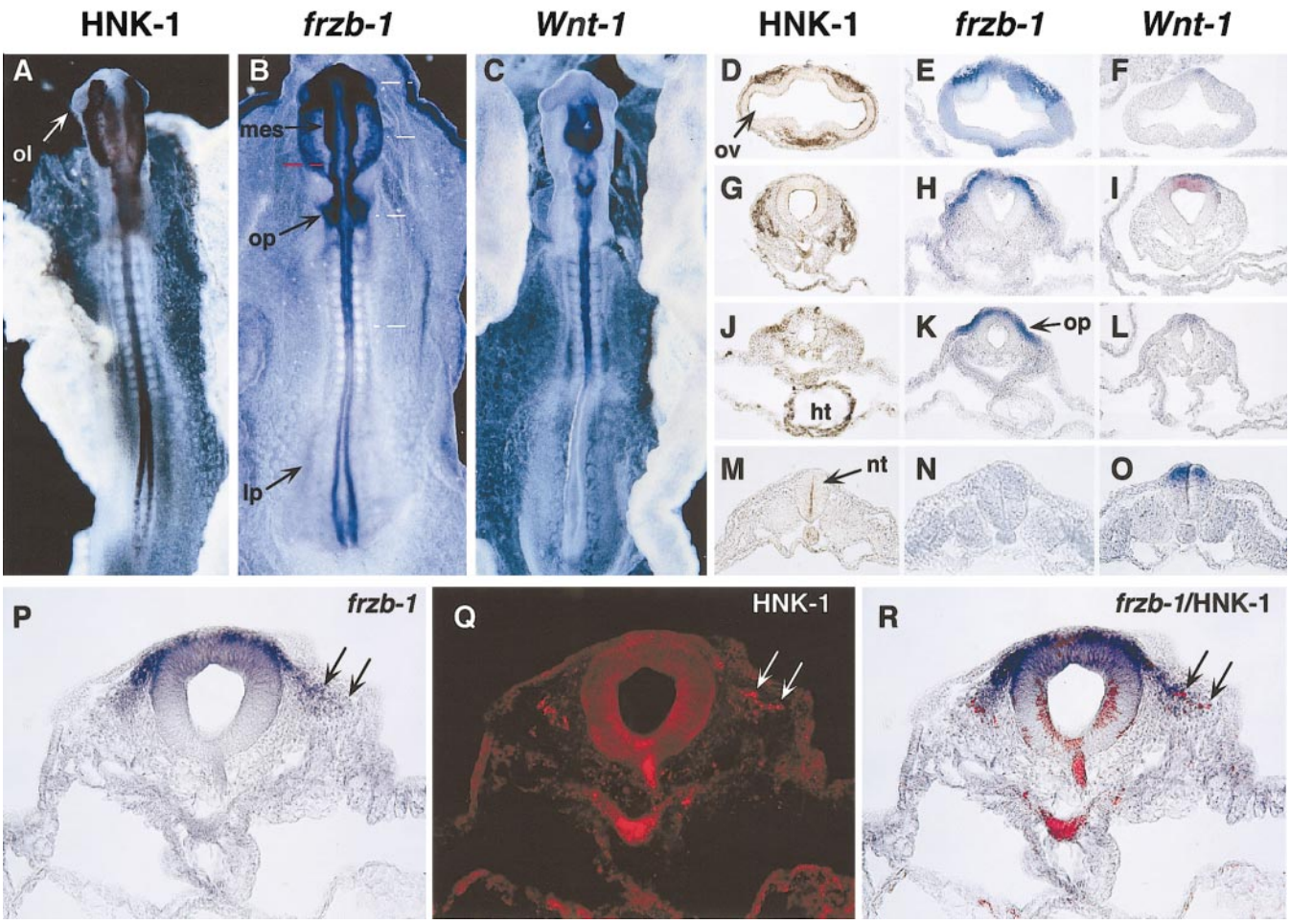

FIG. 5. Comparison of the expression patterns of HNK-1, *cfrzb-1,* and *Wnt-1* in stage 10/11 embryos. Embryos were labeled in whole mount by immunostaining (HNK-1; A) or *in situ* hybridization (*cfrzb-1,* B; *Wnt-1,* C). Representative sections through the forebrain (D, E, and F), the mesencephalon (G, H, and I), the rhombencephalon (J, K, and L), and the trunk (M, N, and O) are shown. The approximate levels of the sections are denoted by the white dashed lines in B. The expression patterns of HNK-1 and *cfrzb-1* are nearly identical except that *cfrzb-1* is more strongly expressed immediately adjacent to the dorsal neural tube. Colocalization of *cfrzb-1* transcripts and HNK-1 epitopes is shown in P, Q, and R. The level of the section shown is denoted by the red dashed line in B. The double arrows point to cells that are clearly labeled for both *cfrzb-1* and HNK-1 expression. Abbreviations: ht, heart; mes, mesencephalon; nt, neural tube; ol, optic lobe; op, otic placode; ov, optic vesicle.

strate that *cfrzb-1* expression is greatly attenuated in the closed neural tube (Figs. 5B and 5N). In direct contrast, *Wnt-1* expression is greatly increased in the same region (Figs. 5C and 5O). This reciprocal pattern of expression between *cfrzb-1,* which is predominantly expressed in cells adjacent to the neural tube, and *Wnt-1,* which is restricted the dorsal neural tube, continues through at least stage 20 (data not shown).

Expression of Chick frzb-1 in Developing Bones

The expression pattern of *cfrzb-1* in limbs (Figs. 8B, 8J, and 8N) correlates well with data from mouse and human embryos (Hoang *et al.,* 1996, 1998). *cfrzb-1* is expressed in both dermis/epidermis (data not shown) and condensing bone primordia of the limb (Figs. 8B, 8J, and 8N). Expression is seen in the condensing mesenchyme at stage 24 (data not shown) and is restricted to the ends of the forming bones by stage 30/31 (Fig. 8J). By stage 30/31, *cfrzb-1* is predominantly expressed at the distal ends of the forming digits, with lower levels seen in more proximal joints (Fig. 8J). To more rigorously determine where *cfrzb-1* is expressed, we compared its expression pattern to that of *sox-9. sox-9* is expressed in cartilage at the start of differentiation (Healy *et al.,* 1996) and is an early regulator of cartilage differentiation (Healy *et al.,* 1999). Although the *cfrzb-1* labeling is not as robust as that of *sox-9,* it is expressed in a pattern virtually identical to that of *sox-9* (Figs. 8I, 8J, 8M, and 8N), suggesting a possible role for *cfrzb-1* in cartilage

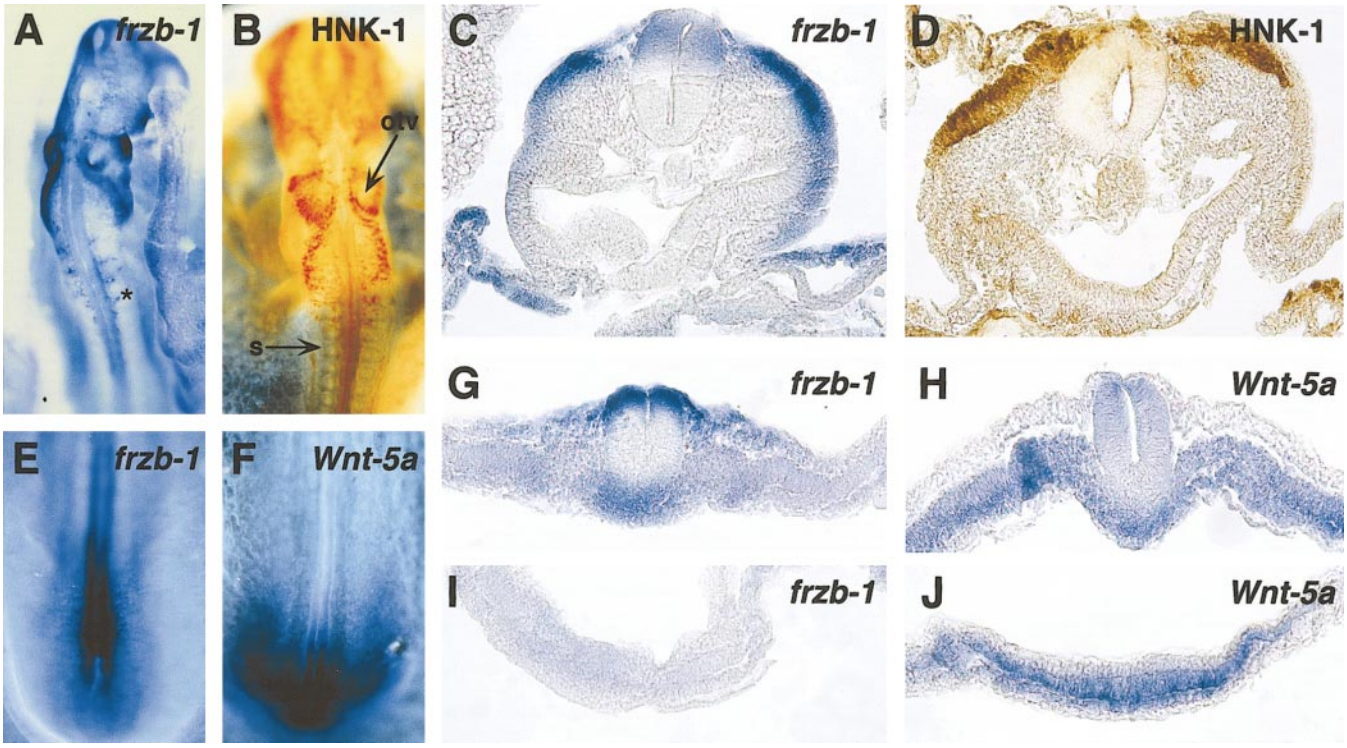

FIG. 6. *cfrzb-1* transcripts and HNK-1 epitopes exhibit virtually identical patterns of expression while *cfrzb-1* and *Wnt-5a* are expressed in reciprocal patterns. Embryos were labeled in whole mount by *in situ* hybridization (*cfrzb-1,* A and E; *Wnt-5a,* F) or immunostaining (HNK-1, B). Sections (15 μm) at the level of the hindbrain are shown for *cfrzb-1*- (C) and HNK-1- (D) labeled embryos. Caudally, sections taken rostral to Hensen's node (G and H) and at the level of the primitive streak (I and J) are shown for both *cfrzb-1* (G and I) and *Wnt-5a* (H and J). Note the characteristic triangular pattern of cells emigrating from the neural tube (see * in A). Abbreviations: otv, otic vesicle; s, somite.

differentiation. As *Wnt-5a* and *Wnt-7a* have both been previously shown to be expressed in developing limbs and to inhibit chondrogenic differentiation, we sought to compare the expression pattern of *cfrzb-1* with both of the *Wnt* genes. Consistent with previously reported data, *Wnt-5a* expression is seen in the apical ectodermal ridge and throughout the developing mesenchyme when limb buds first emerge (Fig. 7C; Dealy *et al.,* 1993). As the limb bud develops, a gradient of *Wnt-5a* expression is seen such that *Wnt-5a* is expressed at low levels in the proximal limb and high levels in the distal limb. Although *cfrzb-1* and *Wnt-5a* expression overlap in the mesenchyme during early stages of limb development, their patterns shift to a more complementary relationship when the mesenchymal condensations that will give rise to bone are formed. Whereas *cfrzb-1* is expressed in the condensing mesenchyme at stage 26, *Wnt-5a* is absent (Kawakami *et al.,* 1999) (Figs. 8J, 8K, 8N, and 8O). Likewise, *Wnt-7a* is expressed at the tips of the digits in a ring that surrounds the *cfrzb-1* expression in the cartilaginous condensation (Fig. 8P).

cfrzb-1 is also expressed in the condensing bone primordia of the vertebrae (Fig. 8G) and ribs (data not shown) in stage 30 embryos. Since vertebrae are derived from the sclerotomal compartment of the somite, it is important to note that *cfrzb-1* is not expressed in the sclerotome of stage 10/11 embryos (Fig. 5N).

Other Areas of cfrzb-1 Expression

By stage 11 of development, both *cfrzb-1* (Fig. 5B) and *Wnt-5a* (data not shown) are expressed in otic placodes and continue to be expressed in the developing otic vesicles through at least stage 20 of development (Figs. 7B and 7C). Expression of both genes appears to be greatly attenuated by day 6 of development (Figs. 8B and 8C). In addition, both *cfrzb-1* and *Wnt-5a* are expressed during development of the eye. Whereas *cfrzb-1* is expressed in the dorsal periphery of the eye at stage 20 of development, *Wnt-5a* transcripts are found around the entire periphery of the eye while being significantly more concentrated ventrally (Figs. 6B and 6C). By stage 26 of development, however, *cfrzb-1* is primarily localized to the surface epithelium of the eye with diminished expression in the corneal epithelium (Fig, 8D). And last, we have discovered that *cfrzb-1* is expressed in the sexually indifferent gonad of the stage 30 embryo (Fig. 8H).

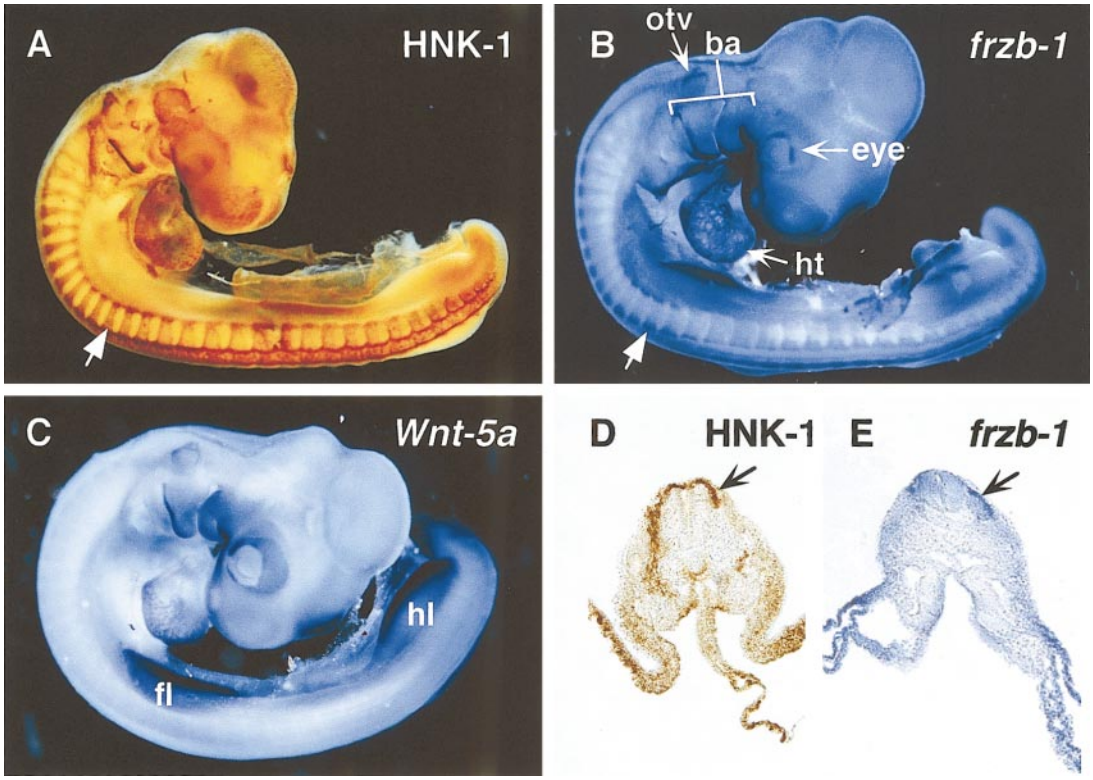

FIG. 7. Distribution of HNK-1, *cfrzb-1,* and *Wnt-5a* in stage 19/20 embryos. Embryos were labeled in whole mount by immunostaining (HNK-1, A) or *in situ* hybridization (*cfrzb-1,* B; *Wnt-5a,* C). All three probes label cells in the branchial arches and the heart. HNK-1 and *cfrzb-1* are expressed in nerve bundles in the anterior half of each somite (see arrowheads in A and B). Representative sections (15 μm) of stage 19 (HNK-1, D) and 14 (*cfrzb-1,* E) embryos are shown to illustrate the migration of neural crest cells down the ventromedial pathway (see arrowheads). Abbreviations: ba, branchial arches; fl, forelimb; hl, hindlimb; ht, heart.

DISCUSSION

We have isolated the chick orthologue of *frzb-1* and analyzed its expression pattern. In many cases, *cfrzb-1* and *Wnt* genes exhibit reciprocal patterns of staining, consistent with the previously proposed model that Frzbs act as antagonists of Wnt proteins.

Chick *frzb-1* is expressed in a pattern complementary to those of *Wnt-3a, Wnt-5a,* and *Wnt-8c* in streak-stage embryos. Whereas *cfrzb-1* is expressed in neural ectoderm beginning when this tissue becomes potent for this fate (Darnell *et al.,* 1999), *Wnt-3a, Wnt-5a, Wnt-5b* (A. McMahon, personal communication), and *Wnt-8c* (Hume and Dodd, 1993) are expressed in the primitive streak. These patterns of complementary expression are reminiscent of the distribution of activities of the organizer back-up system (Psychoyos and Stern, 1996; Yuan *et al.,* 1995a,b), with *Wnts* expressed in the region capable of inducing an organizer (perhaps in response to cVg1; Shah *et al.,* 1997) and *cfrzb-1* expressed in the region competent to respond to these organizer-inducing signals. If this correlation is meaningful, then one would expect that Wnts could be involved in node or notochord induction. In the mouse, as in the chick, *Wnt-3a, Wnt-5a,* and *Wnt-5b* are expressed in overlapping domains of the primitive streak mesoderm (Takada *et al.,* 1994). Although these genes may be functionally redundant in regions in which there is overlapping expression, targeted disruptions of the *Wnt-3a* (Takada *et al.,* 1994) and *Wnt-5a* (Yamaguchi *et al.,* 1999) genes have revealed distinct phenotypes. Mice homozygous for null alleles of *Wnt-3a* exhibit defects in somites, notochord, and neural tube, consistent with the hypothesis that the normal function of the node in these embryos has been disrupted. Mice with mutated *Wnt-5a* alleles exhibit defects in the elongation of the A/P axis, suggesting a proliferative role for *Wnt-5a* or a disruption of the organizer's role in axis extension. If Wnt-3a and Wnt-5a are involved in organizer induction, this suggests that cFrzb-1 could be involved in inhibiting ectopic node induction in the competent neuroectoderm of the normal gastrula. Alternatively, Wnt-Frzb antagonism may play a role in specification of the A/P axis (Yamaguchi *et al.,* 1999) or in the specification of paraxial mesodermal cell fates from neuroectoderm during gastrulation (Yoshikawa *et al.,* 1997).

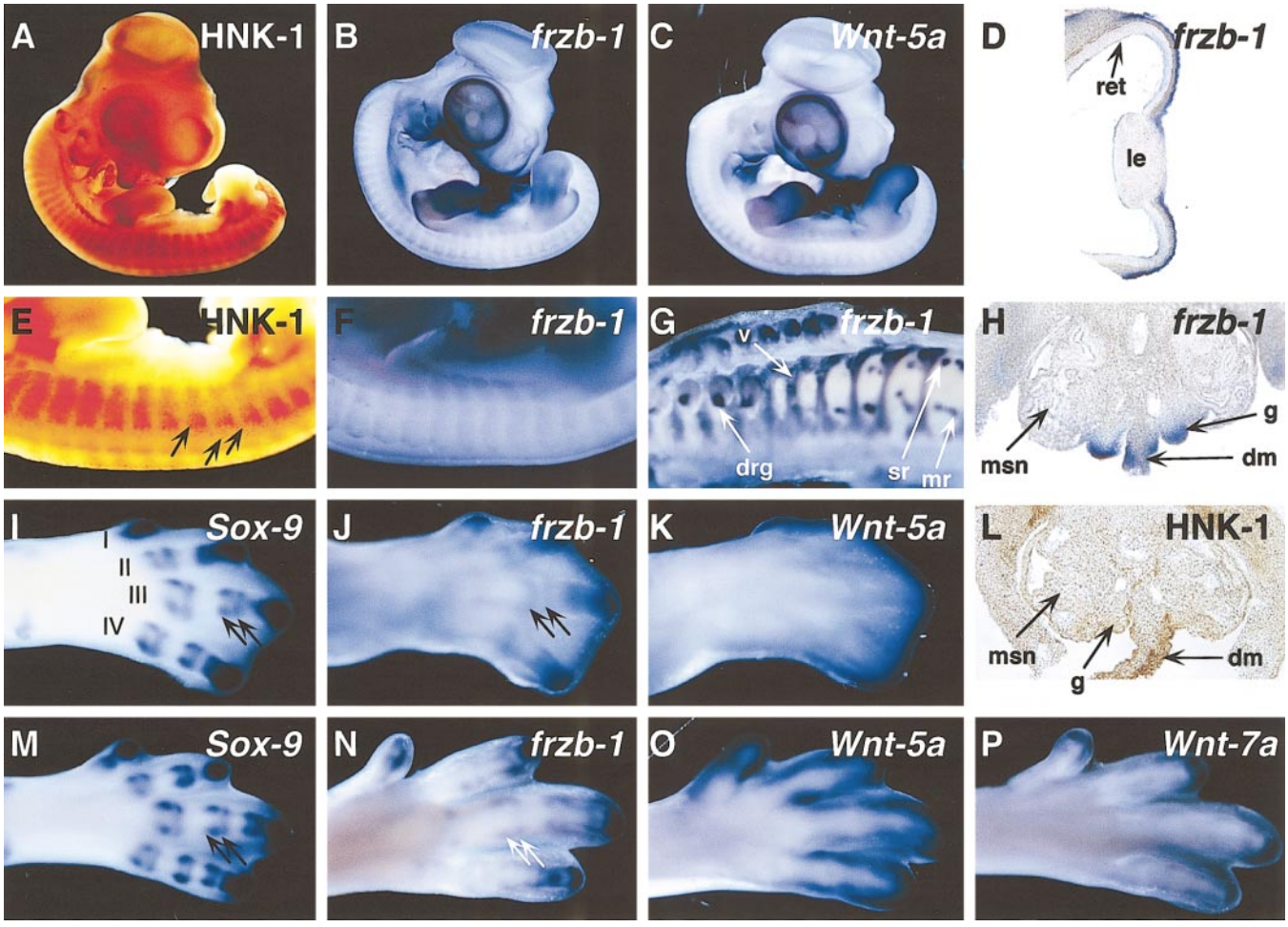

FIG. 8. Localization of HNK-1, *cfrzb-1*, *Wnt-5a*, *Wnt-7a*, and *sox-9* in whole mounts and sections. Whole-mount labeling of stage 26 embryos is shown for HNK-1, *cfrzb-1*, and *Wnt-5a* (A–C) with enlarged views of HNK-1 and *cfrzb-1* labeling in the trunk shown in (E) and (F). A punctate pattern is evident in neural crest cells migrating through the dorsolateral pathway in HNK-1-labeled embryos (E), but not in *cfrzb-1*-labeled embryos (F). A cross section through a stage 26 eye shows *cfrzb-1* expression in the surface epithelium of the eye that is diminished in the corneal epithelium (D). A comparison of *sox-9*, *cfrzb-1*, and *Wnt-5a* expression in stage 30/31 hindlimbs is shown in (I), (J), and (K), respectively (with the digits labeled I–IV), while expression of these genes and *Wnt-7a* in a stage 33/34 hindlimbs is shown in (M), (N), (O), and (P). The double arrows in M and N point to cartilaginous condensations on both sides of the joint. Sections (15 μm) through stage 26 embryos show expression of both HNK-1 (L) and *cfrzb-1* (H) in the dorsal mesentery and *cfrzb-1* in the developing gonad. The neural tube and adjacent structures at the cervical level were dissected and subjected to whole-mount *in situ* hybridization with a *cfrzb-1* probe (G). *cfrzb-1* is clearly expressed in both vertebrae and neural crest cell-derived structures. Abbreviations: dm, dorsal mesentery; drg, dorsal root ganglia; g, gonad; le, lens; msn, mesonephros; mr, motor roots; ret, retina; sr, sensory roots; v, vertebrae.

In *Xenopus*, *cerberus* is expressed in the anterior endoderm and is required for the formation of the head (Piccolo *et al.*, 1999). Interestingly, Cerberus antagonizes BMP, Wnt, and Nodal signals, leading the authors to propose that inhibition of signals required for trunk development is necessary in order for head formation to occur. By analogy with the mouse model system, we think that it is likely that Wnt-3a and Wnt-5a could be involved in trunk development and that cFrzb-1 may play a role in the inhibition of these signals in the head. In both of these scenarios, we would expect that cFrzb-1 would be able to both bind to and inhibit the activity of Wnt-3a and/or Wnt-5a. It has been reported that Frzb-1 is unable to inhibit either Wnt-3a or Wnt-5a from inducing *siamois* and *Xnr3* in a *Xenopus* assay (Lin *et al.*, 1997; Wang *et al.*, 1997b). However, we do not know the molar stoichiometry of the Wnt, Frzb, and Frizzled proteins in this assay, so it is impossible to draw any definitive conclusions. Until soluble, active, purified Frzb-1 and Wnt proteins become available and the *in vivo* concentrations of these proteins

have been determined, it will be difficult to unambiguously determine the binding specificities and inhibitory activities of various Frzb family members.

As Wnt proteins have demonstrated roles in the proliferation and specification of neural crest cell lineages (Augustine *et al.*, 1993; Chang and Hemmati-Brivanlou, 1998; Dorsky *et al.*, 1998; Ikeya *et al.*, 1997; LaBonne and Bronner-Fraser, 1998; Saint-Jeannet *et al.*, 1997), we found the expression of *cfrzb-1* in neural crest cell lineages to be of particular interest. At cranial levels, neural crest cells emerge from the dorsal neural tube of the caudal forebrain, the midbrain, and the hindbrain to contribute to structures of the head, including bones of the face, ciliary ganglia, and connective tissue of the eye (Le Douarin, 1982). At trunk levels, neural crest cells emerge from the dorsal neural tube and follow one of two pathways: the ventromedial pathway, which contributes to the neurons and glial cells of the peripheral nervous system, and the dorsolateral pathway, which gives rise to the melanocytes of the skin. Expression of *cfrzb-1* in cranial regions is consistent with previously reported data from the mouse (Hoang *et al.*, 1998). Comparison of staining patterns for HNK-1, a known marker for migrating neural crest cells, and *cfrzb-1* suggests that *cfrzb-1* is expressed in all HNK-1-positive crest cell lineages in the cranial region. However, in the trunk, the observed pattern of staining is consistent with *cfrzb-1* being expressed in the ventromedial pathway of migration, but not the dorsolateral pathway. In both mouse and chick, *Wnt-1* and *Wnt-3a* are expressed in the dorsal neural tube (Hollyday *et al.*, 1995; Parr *et al.*, 1993; Roelink and Nusse, 1991; Shackleford and Varmus, 1987), the region from which crest cells emigrate. Recently, it has been shown that mice lacking functional alleles for both Wnt-1 and Wnt-3a exhibit a general decrease in the overall numbers of neural crest cells as well as a virtually complete loss of cells in the dorsolateral pathway (Ikeya *et al.*, 1997). These data suggest that Wnt-1 and Wnt-3a have a mild proliferative effect on neural crest cell lineages and that they are absolutely required for either migration or specification of dorsolateral cells. These data are consistent with data obtained in zebrafish which demonstrated that ectopic expression of *Wnt-1* was sufficient to convert neuronal crest cell lineages into pigment crest cell lineages, and inhibition of the Wnt signaling pathway was sufficient to convert pigment lineages into neuronal lineages (Dorsky *et al.*, 1998). As Frzb-1 has previously been shown to inhibit the activity of Wnt-1 (Finch *et al.*, 1997; Leyns *et al.*, 1997; Lin *et al.*, 1997; Mayr *et al.*, 1997; Wang *et al.*, 1997a; Xu *et al.*, 1998), the expression of *cfrzb-1* in the ventromedial pathway, but not the dorsolateral pathway, suggests the exciting possibility that it may play an important role in neural crest specification by inhibiting Wnt signals in the ventromedial pathway.

However, as we do observe faint staining for *cfrzb-1* in the dermis/epidermis, we cannot rule out the possibility that neural crest cells migrating down both ventromedial and dorsolateral pathways are exposed to Frzb protein. As a proliferative role for Wnt-1 and Wnt-3a has also been suggested (Ikeya *et al.*, 1997), it is possible that cFrzb-1 may play a role in crest cells as they make a transition from proliferation to differentiation. In addition, as Wnts have known effects on cell adhesion (Olson *et al.*, 1991; Shibamoto *et al.*, 1998), it is possible that expression of cFrzb-1 in migrating crest cells is required for the delamination of crest cells from the neural tube.

The spatiotemporal expression pattern of *cfrzb-1* in developing bones also suggests a potential role in chondrogenesis. Frzb-1 protein was originally purified from articular cartilage extracts from cows based on its ability to stimulate chondrogenesis (Hoang *et al.*, 1996). In conjunction with the demonstrated ability of Wnt-1, Wnt-5a, and Wnt-7a to inhibit chondrogenic differentiation (Kawakami *et al.*, 1999; Rudnicki and Brown, 1997; Stott *et al.*, 1999; Zakany and Duboule, 1993) as well as the ability of Wnt-1 to inhibit specification of sclerotomal cell fates in developing somites (Capdevila *et al.*, 1998), these data suggest that Frzb-1 may act by antagonizing Wnt activity. Consistent with previous analysis of *frzb-1* expression in mouse (Hoang *et al.*, 1998) and human (Hoang *et al.*, 1996), *cfrzb-1* is expressed in the condensing bone primordia in the limb. However, in contrast to the expression of *frzb-1* in human embryos (Hoang *et al.*, 1996), our data also indicate that *cfrzb-1* is expressed in cartilaginous vertebrae, suggesting a chondrogenic role for *cfrzb-1* outside of the limb.

During bone development, *cfrzb-1* is expressed in domains adjacent to both *Wnt-5a* and *Wnt-7a*. In addition to its ability to inhibit chondrogenic differentiation (Kawakami *et al.*, 1999), recent experiments suggest that *Wnt-5a* has a proliferative capacity in the developing limb (Yamaguchi *et al.*, 1999). Thus, the complementary patterns of expression for *Wnt-5a* and *cfrzb-1* suggest that *cfrzb-1* may be expressed in cells that are undergoing a transition from proliferation to differentiation.

Finally, the expression of *cfrzb-1* in the gonad suggests the possibility that *cfrzb-1* might also play a role in either sex determination or proper patterning of the gonad. Data from the mouse, in which Wnt-4 is required for female sexual development (Vainio *et al.*, 1999) and Wnt-7a is required for proper uterine patterning (Miller and Sassoon, 1998), are consistent with this possibility.

Our data suggest that cFrzb-1 is likely to play multiple roles in chick development. Although Frzb proteins may be able to modulate the activity of Wnt proteins in a number of ways, our comparison of the expression pattern of *cfrzb-1* with expression patterns and known functions of Wnt proteins leads us to propose that cFrzb-1 is acting as an antagonist of Wnt activity in gastrulation, neural crest cell specification, and chondrogenesis in the limb. To date, the expression patterns for fewer than one-half of the known vertebrate Wnts have been published. Thus, it is possible that other roles for cFrzb-1 will be suggested as the expression patterns and activities of additional Wnt family members become known. In addition, although there are not yet any indications of specificity in Frzb/Wnt binding, it seems

likely that this will be an important regulatory feature as there is evidence for specificity of binding and activity between *Drosophila* Wnts and Frizzled proteins (Bhanot *et al.*, 1996).

cFrzb-1 appears to belong to an emerging class of signaling-molecule inhibitors. Other examples of secretory molecules with inhibitory action include noggin, follistatin, and chordin, all of which inhibit BMPs (for review, see Smith and Harland, 1992); secreted Hedgehog-interacting protein, which inhibits Sonic hedgehog (Chuang and McMahon, 1999); and Argos, which is an inhibitor of *Drosophila* Spitz (Schweitzer *et al.*, 1995). Recently, two additional inhibitors of Wnt activity, Dickkopf and WIF-1, have been described (Glinka *et al.*, 1998; Hsieh *et al.*, 1999). The expression of *Wif-1* in neural crest cell derivatives suggests that functional redundancy of Frzb-1 and WIF-1 may be required to ensure proper establishment and patterning of the peripheral nervous system. These inhibitory interactions appear to be important for establishing and maintaining distinct boundaries and present a recurring mode of regulation during embryonic development. The expression pattern of *cfrzb-1* is consistent with it having this type of role and our analysis has generated several testable hypotheses about the potential functions of cFrzb-1 that will enable us to pursue functional studies.

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Note added in proof. Since the original submission of our manuscript, we have become aware of similar studies performed in the Francis-West laboratory at Guy's Hospital in London (R. K. Ladher, V. L. Church, S. Allen, L. Robson, N. A. Brown, G. Hattersley, V. Rosen, F. P. Luyten, L. Dale, and P. H. Francis-West). Cloning and expression of the Wnt antagonists Sfrp-2 and Frzb during chick development (Manuscript submitted). Their data describing chick *frzb-1* expression are consistent with ours with only one exception: They report *cfrzb-1* expression in neural crest cells migrating down the dorso-lateral pathway in the trunk, whereas we were unable to detect any expression in this cell population.

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